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Original Article

Changes of androgen receptor expression in stages VII-VIII seminiferous tubules of rat testis after exposure to methamphetamine

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Abstract

Methamphetamine (METH) is toxic not only to the central nervous system but also to the reproductive system. This study aimed to investigate the effect of METH administration on androgen receptor (AR) expression in the seminiferous tubules (stages VII-VIII) and Leydig cells. 24 Sprague-Dawley rats were equally divided into 4 groups; control, acute dose-METH binge (AB-METH), escalating dose-METH (ED-METH) and escalating dose-METH binge (ED-METH binge) groups. The expressions of AR were examined using immunohistochemical staining. AR expressions in round and elongated spermatids showed significant decreases in ED-METH binge and AB-METH groups. In Leydig's cells, AR expressions were significantly decreased in both ED-METH and ED-METH binge groups. These results could reflect the effect of METH on the expressions of AR in both round and elongated spermatids of stages VII-VIII seminiferous tubule and Leydig's cells. Decreases of AR expressions in those cells of METH-treated animals may interrupt spermatogenesis at these stages.

Keywords: methamphetamine, seminiferous tubule, androgen receptor, spermatogenesis, testis

1. Introduction

1.1 Methamphetamine and reproductive system

Methamphetamine (METH), a central nervous system toxic stimulant, has also been reported to have an effect on the male reproductive system. It has been reported that testosterone with its signal through AR is vital for complete spermatogenesis (O'Hara and Smith, 2015). Additionally, the actions of both testosterone and AR on spermatogenesis are mediated by Sertoli cells attaching to maturing germ cells (O'Hara and Smith, 2015). Yamamoto *et al*. (1999) has studied the effect of METH on male mice. The study showed that METH at 15 mg/kg induces a decrease of sperm motility. In 2002, the study of Yamamoto and co-workers also revealed that METH at 5, 10 and 15 mg/kg induces apoptosis of cells in the seminiferous tubules. Moreover, METH at 10 and 15 mg/kg can induce fluctuations of serum testosterone concentration. Additionally, METH at 1, 5 and 10 mg/kg can induce a decrease of proliferation/apoptosis in spermatogonia (Alavi *et al*., 2008). Our previous study also demonstrated that METH induces abnormal sperm morphology, decreases sperm concentration and induces apoptosis of cells in the seminiferous tubules (Nudmamud-Thanoi and Thanoi, 2011).

1.2 The stage of the cycle of the seminiferous epithelium in the rat

The stage of the cycle of the seminiferous epithelium in the rat can be divided into 14 stages (stage I-XIV). Through the 14 stages of the seminiferous epithelium, the spermatogonia will be changed into spermatozoa when the process completed (Leblond and Clermont, 1952). The stages VII-VIII seminiferous epitheliums in rat are known as the androgen-

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dependent stages because these stages show the highest levels of androgen receptor expression (Lue *et al*., 2000). Consequently, the expression of AR in the nucleus of Sertoli cell is also highest at these stages (Hill *et al*., 2004).

The previous study of O'Donnell *et al*. (1996) suggested that testosterone is necessary for spermatogenesis at stage VII-VIII. Suppression of testosterone at these stages can inhibit the development of round spermatids. Therefore, this study aimed to investigate the changes of AR expression in seminiferous epithelium at stages VII-VIII in male rats treated with METH consumption in comparison with a control group.

2. Materials and Methods

2.1 Animals

Twenty-four adult male Sprague-Dawley rats (8-week old) from National Laboratory Animal Center, Mahidol University, Nakorn Pathom, Thailand weighing between 200-250 g were housed one per cage at 24±1°c and dark/light cycle 12 h. Experimental protocols for this study were approved by the Animal Research Committee of Naresuan University, Thailand (NU-AE530615; NU-AE540534). All animals were divided into 4 groups with 6 animals each, including control group, acute dose-METH binge (AB-METH) group, escalating dose-METH (ED-METH) group and escalating dose-METH binge (ED-METH binge) group. The last two groups imitated METH addiction in human (Segal *et al*., 2003).

2.2 Methamphetamine administration

D-methamphetamine hydrochloride (Lipomed AG, Arlesheim, Switzerland) with the consent from the Ministry of Public Health was used in this experiment. The animals were treated with METH or saline by intraperitoneal (i.p.) injection. In the control group, animals were treated with 0.9% saline at 3 h interval for 14 days. On day 15, animals were treated with 0.9% saline every 2 h 4 times before being sacrificed. In the AB-METH group, animals were treated with 0.9% saline at 3 h interval for 14 days. On day 15, animals were treated with METH 6 mg/kg at 2 h intervals 4 times before being sacrificed. In the ED-METH group, animals were treated with METH with gradual increasing concentration from 0.1 mg/kg to 3.9 mg/kg every 3 h on day 1 to day 13. Then, METH at 4.0 mg/kg was injected every 3 h interval on day 14. On day 15, animals were treated with 0.9% saline every 2 h for 4 times interval before sacrificed. In the ED-METH binge group, animals were treated with METH the same as in the ED-METH group on day 1 to day 14 but animals were treated with METH 6 mg/kg every 2 h 4 times before being sacrificed on day 15. All animals were sacrificed by cervical dislocation. All examined organs, including testes were removed gently and kept in 10% formalin for further studies.

2.3 Tissue preparation

After removal, testes were immediately immersed in 10% formalin at least 3 days to preserve tissue structure. Testes from each group were cut and processed for paraffin embedding. The tissue blocks were kept at 4°C until sectioning. Tissue blocks were coronally sectioned using a microtome at 5 μm thickness. The sections were floated on warm water at 45°C before mounting onto silane coated slides. Tissue sections were allowed to dry at room temperature overnight.

2.4 Immunohistochemistry study

Immunohistochemistry study is used for localization of androgen receptor (AR) expression on tissue. Paraffin sections were deparaffinized by xylene 2 times for 5 min and rehydrated by gradual series of alcohol dilution (100%, 95%, 80%, 70%, respectively) to distilled water for 5 min. After that, antigen was retrieved with high temperature heating in a microwave oven 3 times for 5 min at 560 Watt and allowed to cool down at room temperature for 30 min. Then, tissue sections were washed in PBS 3 times for 5 min each and incubated for 30 min with endogenous peroxidase blocking solution including 10% methanol, 0.3% hydrogen peroxide (H_2O_2) and 1% triton X diluted in PBS and washing with PBS 3 times for 5 min. Non-specific proteins were blocked with 5% normal goat serum (Vector Laboratories, Burlingame, California, USA) diluted in PBS for 2 h. Then, tissue sections were incubated with anti-androgen receptor primary antibody (Santa Cruz Biotechnology, Santa Cruz, California, USA) 1:25 diluted with PBS containing 5% normal goat serum overnight at 4°C following washing with PBS 3 times for 5 min.

Biotinylated secondary antibody and avidin-biotinylated horseradish peroxidase complexes (ABC kit) (Vector Laboratories, Burlingame, California, USA) were used at 1:200 dilution in PBS containing 5% normal goat serum for 2 h and washed with PBS 3 times for 5 min. The ABC kit was prepared 30 min before use and tissue sections were incubated with ABC kit for 60 min and washed with PBS 3 times for 5 min. Eventually, tissue sections were incubated with 3,3'-Diaminobenzidine (DAB) (Vector Laboratories, Burlingame, California, USA) for 15 min to visualize androgen receptor protein on tissue sections. The reactions between tissue sections and DAB were stopped by distilled water for 5 min. After that, tissue sections were counterstained with hematoxylin 2 dips and rinsed with tap water for 5 min. Then, tissue sections were dehydrated by gradual series of alcohol concentration (70%, 80%, 95%, 100%, respectively) and by xylene 2 times for 5 min each to remove alcohol. Mounting medium (Fisher scientific, New Jersey, USA) was used and tissue sections were allowed to dry at room temperature overnight.

Each tissue section was observed under a Nikon eclipse 80i microscope (Hollywood International Ltd., BKK, Thailand) and seminiferous tubules at stages VII-VIII from each group were photographed with Nikon DXM1200c.

2.5 Evaluation of immunoreactive cell

Twenty images of stages VII-VIII of seminiferous tubules and Leydig cells around stage VII-VIII of seminiferous tubules were chosen randomly from two different tissue sections of each rat and examined under bright field microscope with 20X objective lens. Identification of stages VII-VIII of seminiferous tubules was based on the study of Hess (1990). Briefly, stage VII was distinguished by the enlargement of the basophilic granule in the cytoplasm of the step 19 spermatid. Another important figure of this stage was the alignment of elongated spermatids along the luminal border. The transition from stage VII to stage VIII was identified by the basophilic granules moved to a location beneath the head of the step 19 spermatid. Additionally, some stage VII-VIII transitions had step 7 spermatids whose nuclei were located centrally within the cytoplasm. AR expression in Sertoli cells, round spermatids, elongated spermatids and Leydig cells were analyzed by Image J Program (http://rsb.info.nih.gov/ij/). AR expression was presented as percentage of AR expression in Sertoli cells, round spermatids and elongated spermatids per total cells of each cell type. AR expression in Leydig cells was counted around stage VII-VIII of seminiferous tubule and presented as percentage of AR expression in Leydig cells per total Leydig cells.

2.6 Statistical analysis

AR expression in Sertoli cells, round spermatids, elongated spermatids and Leydig cells was analyzed by one-way ANOVA following by Dunnett's post hoc test on SPSS program version 11.5. The data were presented as mean ± SEM. The significance was defined as p-value less than 0.05.

3. Results

Immunohistostaining demonstrated that AR expressions were observed in all groups examined. Generally, AR were expressed in Sertoli cells, round and elongated spermatids of seminiferous epithelium at stages VII-VIII and Leydig cells around the tubules (Figure 1)

3.1 Androgen receptor expression in spermatids

The percentages of AR expressions in round spermatids were significantly decreased ($p<0.05$) in the ED-METH group (89.56±3.55%) and ED-METH binge group (88.52± 11.19%) when compared with the control group (99.31± 0.21%) (Figure 2). The percentage of AR expression in elongated spermatids was significantly decreased only in the AB-METH group (60.89±5.10%) ($p<0.001$) when compared with the control group (84.02±2.53%) (Figure 3).

3.2 Androgen receptor expression in Leydig cells and Sertoli cells

Percentages of androgen receptor (AR) expression in Leydig cells was significantly decreased in the ED-METH group (74.54±1.49%) ($p<0.05$) and the ED-METH binge group (70.72±2.15%) ($p<0.001$) when compared with the control group (80.48±1.60%) (Figure 4). However, there were no differences in the percentages of AR expression in Sertoli cells in all METH-treated groups when compared with the control group (Figure 5).

4. Discussion

The expression of AR in round spermatids at stages VII-VIII seminiferous epithelium was decreased in ED-METH and ED-METH binge groups while the expression of AR in

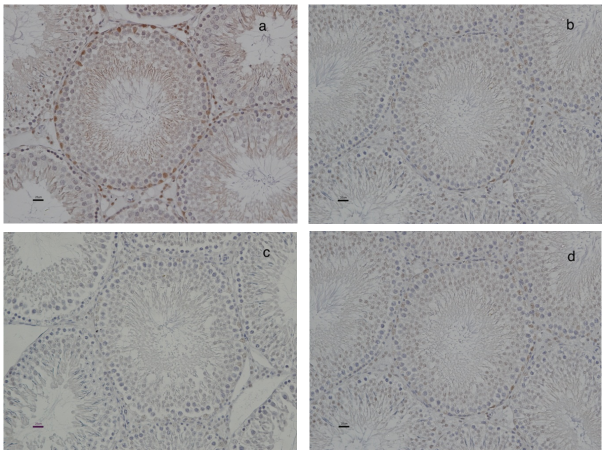

Figure 1. Immunohistostaining of AR expression in stages VII-VIII of seminiferous epithelium in testes of rats in the control group (a), AB-METH (b), ED-METH (c) and ED-METH Binge (d). AR Immunoreactive cells (stained brown) were expressed in Sertoli cells, round and elongated spermatids and Leydig cells in both control and METH-treated groups.

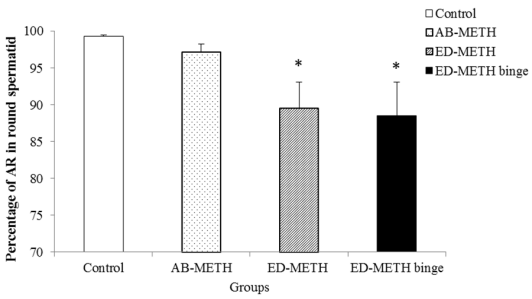

Figure 2. Percentages of AR expression in round spermatids in the control, AB-METH, ED-METH and ED-METH groups. Data are presented as mean ± SEM, n = 6, *$p<0.05$ compared with control group ANOVA post-hoc LSD.

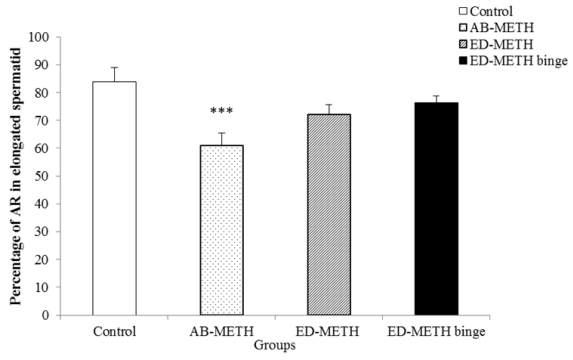

Figure 3. Percentages of AR expression in elongated spermatids in the control, AB-METH, ED-METH and ED-METH groups. Data are presented as mean ± SEM, n = 6, ***p<0.001 compared with control group ANOVA post-hoc LSD.

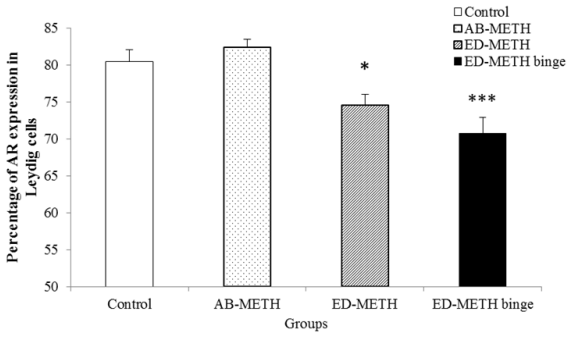

Figure 4. Percentages of AR expression in Leydig cells in the control, AB-METH, ED-METH and ED-METH groups. Data are presented as mean ± SEM, n = 6, *p<0.05 and ***p<0.001 compared with control group ANOVA post-hoc LSD.

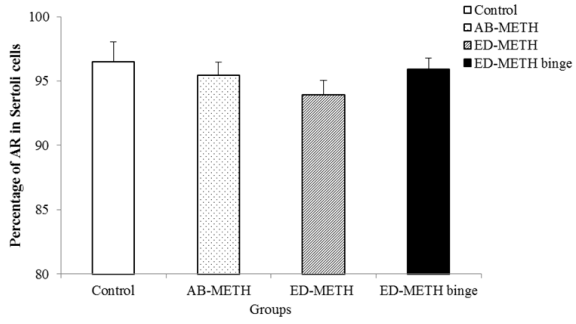

Figure 5. Percentages of AR expression in Sertoli cells in the control, AB-METH, ED-METH and ED-METH groups. Data are presented as mean ± SEM, n = 6, ANOVA post-hoc LSD.

elongated spermatids was decreased in AB-METH group. These results could reflect the interruption in development of spermatids due to the decrease of AR expression in METH-treated rats as it has been generally detected in the nucleus of elongated spermatid of rat (Vornberger *et al*., 1994). Additionally, AR is required for the normal spermatogenesis (Holdcraft and Braun, 2004) and it has been reported as a necessity during meiosis I, transition of round spermatids into elongated spermatids and during spermiogenesis at terminal stage (Xu *et al*., 2007). Moreover, it has been reported that AR expression was depended upon the level of the seminiferous tubule impairment. In tubules with normal spermatogenesis, the AR protein expression was at normal level while seminiferous tubules with an arrest of spermatogenesis, the AR expression was lower (Walczak-Jedrzejowska *et al*., 2013). Decrease of AR expression in round and elongated spermatids may cause the blockage of the transition from round spermatids into elongated spermatids and abnormality at maturation stage of spermiogenesis.

The expression of AR in Leydig cells was decreased in ED-METH and ED-METH binge groups. A decrease of AR expression in Leydig cells is consistent with the previous study which indicated that male mice lacking AR in Leydig cells show that the spermatogenesis was arrested at round spermatids, apoptotic spermatocytes were found in the seminiferous tubules and the serum testosterone level was decreased (Xu *et al*., 2007). In the present study, a decrease of AR in Leydig cells may interrupt hormone synthesis especially testosterone, leading to suppression of spermatogenesis.

The expression of AR in Sertoli cells in the present study was not changed in all groups treated with METH. The previous report was showed that the expression of AR in Sertoli cells was highest at stage VII-VIII of rat seminiferous tubule (Shan *et al*., 1997). Moreover, intensity of AR level was highest in these stages (Tirado *et al*., 2003). AR expression in Sertoli cells is necessary for spermatogenesis particularly in meiosis division of primary spermatocyte and spermiation of spermatid (Holdcraft and Braun, 2004). Additionally, it has been suggested that spermatogenesis in testis may be maintained by reactivity to testosterone in Sertoli cells and AR expression in Sertoli cells has been reported to increase with stimulation of follicle-stimulating hormone (FSH) secretion (Okuyama *et al*., 2014). Therefore, an unchanged of AR expression in the Sertoli cells in METH-treated animals may reflect the compensatory effect of androgen receptor in Sertoli cells in maintaining the proper environment for spermatogenesis.

In conclusion, METH administration can affect the expression of AR in both round and elongated spermatids of stages VII-VIII seminiferous tubule as well as in Leydig cells around the tubules. Decreases of AR expression in those cells of METH-treated animals may reflect an impairment of spermatogenesis at these stages, while an unchanged AR expression in the Sertoli cells may play a critical role to provide the proper environment for spermatogenesis.

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